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The Effects of Phosphate and Hydrogen Ions on the Velocity-pCa Relationship in a Motility Assay
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During intense muscular fatigue elevated levels of inorganic phosphate (P_i) and hydrogen ions (H^+) are believed to inhibit contraction. Evidence suggests that a portion of the inhibition is due to a direct effect on myosin however the most pronounced losses in muscle force are believed to be mediated through the regulatory proteins Troponin (Tn) and Tropomyosin (Tm) indirectly affecting the actomyosin interaction. To begin to understand the molecular basis of this indirect inhibition we determined the effects of H^+ and Pi on velocity-pCa relationship of regulated thin filaments in an *in vitro* motility assay using chicken skeletal myosin. Decreasing pH from 7.4 to 6.5 significantly ($p<0.05$) decreased filament velocity (V_{actin}) saturating Ca^{2+} (pCa 4) from 6.2 ± 0.3um/s to 0.4 ± 0.2 at pH 6.5. In addition, low pH increased the Ca^{2+} level required to reach half-maximal velocity (pCa50) from pCa 6.26 to 5.45, suggesting a pronounced decrease in Ca^{2+}-sensitivity. The addition of 30mM Pi at pH 7.4 caused a minor increase in V_{actin} in the absence of Ca^{2+} (pCa 10), that was dependent on the presence of excess Tn/Tm in the buffer, but these effects were not present at higher Ca^{2+} levels (pCa <7.0). In contrast to pH 7.4, the addition of P_i at pH 6.5 increased V_{actin} at every Ca^{2+} from pCa 9 to pCa 4, and increased the pCa50 5.45 to 7.23 indicating a heighted sensitivity to Ca^{2+}. In addition, at saturating Ca^{2+} adding 30mM Pi increased V_{actin} from 0.4 ± 0.2 to 2.6 ± 0.3 at pH 6.5. These data indicate that H^+ alone can profoundly reduce the Ca^{2+}-sensitivity of thin filaments but that a simultaneous increase in P_i, as occurs during fatigue can counteract these depressive effects on regulated filament velocity *in vitro*.

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Calcium Dependence of Titin-Regulated Passive Forces in Skeletal Muscle Fibers
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There is evidence that the passive forces produced by titin in skeletal muscles may be regulated by Ca2+. Studies have shown an upwards shift in the passive force-sarcomere length (SL) relation when muscle fibres are tested with a high concentration of Ca2+ and the myosin-actin interaction is abolished. In this study we tested the hypothesis that there is a direct relation between the concentrations of Ca2+ and the cross-bridge independent increase in passive forces. Single fibres were isolated from the rabbit psoas muscle and transferred into an experimental chamber, between a force transducer and a motor arm. Fibres were activated in a range of Ca2+ concentrations (pCa2+ between 4.5 and 9.0), before and after administration of the myosin inhibitor blebbistatin, which caused the maximal isometric force to decrease by 93.5%. After blebbistatin administration, the fibres were submitted to a protocol in which they underwent consecutive step-stretches, starting at an initial SL of 2.5μm (amplitude of stretch: 5% initial SL, duration 300 ms, pauses between stretches: 30 sec). We observed an upwards shift in the passive force-SL relation when pCa2+ was lower than 6.4 (increase from 1.06 mN/mm2 to 4.28 mN/mm2 at SL of 2.8μm and from 14.99 mN/mm2 to 27.85 mN/mm2 at SL of 3.8μm). Decreasing the pCa2+ below 6.4 did not change the forces further. The results suggest that there is not a direct relation between passive forces and Ca2+ concentrations; instead there is a threshold for Ca2+ regulation of passive forces via titin (in the condition investigated, a pCa2+ of 6.4), beyond which force does not change significantly.

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Dysferlin is Dispensable for Recovering Saponin-Induced Membrnane Damage but Essential for Recovering Lengthening-Contraction-Induced Injury in mdx Mice
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Dysferlin plays an important role in repairing membrane damage and dysferlin deficiency causes muscular dystrophy. Proteins such as perforin, complement component C9, and bacteria-derived cytolysins, as well as the natural detergent saponin can form large pores on the cell membrane via complexation with cholesterol. However, it is not clear whether dysferlin plays a role in repairing membrane damage induced by pore-forming reagents. Here we showed that dysferlin-deficient muscles recovered the tetanic force production to the same extent as their WT counterparts following a 5-min saponin exposure (50 μg/ml). Interestingly, the slow soleus muscles recovered significantly better than the fast *extensor digitorum longus* (EDL) muscles. These data suggest that dysferlin is unlikely involved in repairing saponin-induced membrane damage, and that the slow muscle is more efficient than the fast muscle in repairing such damage. Furthermore, the mice deficient in both dystrophin and dysferlin (DD-null) exhibited more severe muscle pathology than either *mdx* or dysferlin-null mice. Although lengthening contractions (LCs) caused similar force deficits regardless of dysferlin expression, we observed that the recovery of force within 45 minutes following LCs was hampered in DD-null muscles compared to *mdx* muscles. These data suggest that dysferlin plays a role in recovering the initial LCs-induced muscle injury of the DGC-compromised muscles. Dystrophin deficiency unmasks the function of dysferlin in membrane repair during LCs, providing a useful assay to evaluate the effectiveness of therapies designed to treat dysferlin deficiency.

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Additive Phenotype of MG53 and Dysferlin Deficiencies in Membrane Repair Function of Skeletal Muscle Fibers
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Dysferlin and MG53 have been implicated as members of the sarcolemma repair machinery. Skeletal muscle fibers isolated from mice lacking expression of one or the other protein display defective membrane repair process following acute injuries. Our previous studies show that MG53 can nucleate the assembly of the membrane repair machinery by facilitating translocation of intracellular vesicles to membrane injury sites. Dysferlin appears to participate in Ca^{2+}-dependent fusion of these vesicles for formation of a repair patch, but dysferlin itself cannot translocate to the injury site in the absence of MG53. To test the complementary function of MG53 and dysferlin in cell membrane repair, we generated a double knockout mouse lacking both MG53 and dysferlin. While serum creatine kinase (CK) levels of dysferlin$^{-/-}$, mg53$^{-/-}$, and wild-type mice were not significantly different from each other, CK levels of the mg53$^{-/-}$dysferlin$^{-/-}$ mice were significantly elevated at resting condition. This suggests a more severe membrane repair defect. Plasma membrane targeted UV-irradiation of isolated skeletal muscle fibers in the presence of a membrane impermeant dye (FM1-43) was used to directly assay membrane repair. This experimental procedure was modified from previously established protocols for a quantitative assessment of the muscle membrane repair capacity. Compared with dysferlin$^{-/-}$ and mg53$^{-/-}$ fibers, significant elevated entry of FM1-43 dye was observed in mg53$^{-/-}$dysferlin$^{-/-}$ muscle following UV-irradiation. These results suggest that the involvement of these two proteins in the membrane repair mechanism is more complicated than that as two points along a linear pathway. The additive effects of MG53 and dysferlin in muscle membrane repair suggest the possibility that these two proteins may either sense different membrane injury signals, or that they may act at different compartments of the sarcolemma membrane (caveolae or transverse-tubule network).

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Conserved Surface Residues of Tropomyosin are Required for Cooperative Activation of Actin by Myosin
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Tropomyosins (Tm) are α-helical coiled-coil proteins that associate end-to-end along the length of actin filaments in muscle and non-muscle eukaryotic cells. Conserved functions include binding and stabilization of actin filaments and cooperative regulation of actomyosin. From a phylogenetic analysis we identified the most conserved Tm residues and tested the hypothesis that residues important for actin-related functions should be conserved (Barua et al., 2011). We introduced Ala mutations at conserved b, c, f surface coiled-coil positions of rat striated αTm. The mutations were grouped according to their positions in the first or second half of periods 2-6 in a 7-period model of Tm's sequence. The E. coli -expressed Tms had an N-terminal Ala-Ser to increase actin affinity. We previously reported that most mutations in the first half of periods 2-6 reduce actin affinity greater than 4-fold. Since mutations in the second halves had a less than 2-fold effect, we postulated the residues may be important for another conserved function, such as myosin regulation. Here we report results of in vitro motility assays to study the effects of mutations in the second halves of periods 2-6 on the cooperative regulation of actomyosin. At surface myosin concentrations that allow maximal velocity of naked actin filaments, the velocity of actin-Tm (A-Tm) filaments was ~40-50% lower. Addition of N-ethylmaleimide-modified myosin S1 (NEM-S1) increased the velocity of A-Tm filaments over that of actin alone, in a concentration-dependent manner, illustrating activation. The velocity of A-Tm(mut) filaments was further inhibited by ~50-80% relative to A-Tm, depending on the mutant. Addition of NEM-S1 increased the velocity. The results indicate that conserved residues in the second half of the periodic repeats are required for normal cooperative regulation of actomyosin by Tm. Supported by NIH.